mDSLM Manual

Start-up procedure

1. Switch on computer.
2. Change objectives before switching on microscope (1., 2.).
3. If you are a using a different chamber from the previous user the stage must be realigned by a member of staff before you continue (11.).
4. Screw chamber in, fill with appropriate medium, switch microscope on and start acquisition software (6., 7.). Check for leaks.
5. If you are using more than one laser line you need to align the different laser lines (3., 4., 9.). If you are using one laser line move on to the next item.
6. Align the appropriate beads (10.).
7. Calibrate your rotation compensation tool if you want record multiple angles of your specimen (13.).
8. Insert your sample.

Shut-down procedure

1. Set Φ to Φ=0, set x stage control to x=0, set z stage control to z=0, set y stage control to y=0. Remove sample. Exit acquisition software.
2. Remove medium and unscrew chamber. Clean chamber and tools to prevent rust.
3. Switch microscope off before removing any objective.
4. Unscrew detection objective and clean it.
5. Shut the computer down at the end of the day.

1.

Illumination objectives and detection objectives:

2.5x (NA 0.06) illumination objective generates more homogeneous light for **10x (NA 0.3) and 20x (NA 0.5) detection objectives**. (If you use the 5x illumination objective for the 20x detection objective the light sheet will be slimmer but only over a short distance in the middle and only slim objects can be imaged with this combination.)

5x (NA 0.16) illumination objective generates more homogeneous light for **40x (NA 0.75) and 63x (not currently available) detection objectives**.

- Only change objectives when the microscope is switched off and the chamber has been taken off.
- Every time you change the detection objective change the finger glove or at least check it has not acquired any holes.

How to fit the laser cable on assembly:

The cable has two discernable ends. The microscope end is just pushed in. To fit the end at the laser unit press button to push end in, but do not move the adjustable screws, which have been adjusted and fixed in the factory.

2.

Objective Pifocs:

The Pifocs vibrate when no objective is mounted but will be still when the objective is on. **Always switch the Pifocs off before changing objectives! In order to do that switch the whole microscope off because the Pifoc power switch is hidden.**

3.
Laser lines:
488 nm diode laser
561 nm DPSS solid-state laser (AOM)
638 nm diode laser
The lasers are not quite stable during the first hour of operation. Switch on the electronics with the lasers and wait 20 min before starting an experiment. Even at 0 % power the lasers emit a bit of light. They can be unclicked and so turned off in the software at random, but it is best to leave the solid-state laser on, otherwise you have to wait 10 min before imaging. A photodiode measures laser power all the time, but the reading is only visible in the window for the electronics box and in the log file.

4.
Transmission light:
- Use 760 nm position
- Do not use a filter
- Intensity can only be controlled by changing the exposure time, laser power does not work for transmission

6-position filter wheel:
Semrock filters, not necessarily available in their catalogue but the Stelzer group has a contact person whose details we should get.
- One position has to stay empty, the others contain bandpass or longpass filters.
- Filter wheel can be turned manually when it is off but never try that when it is on.
- Ludl Electronics filter wheel controller with green on/off button
- With time filters will become loose and start to rattle and need to be tightened up again.
- The filter wheel must not touch the microscope to avoid vibration. If the filter wheel does touch the microscope casing you will get vibration visible by the generation of sinus waves on screen and the filter wheel position will have to be adjusted.

The air table needs to be well aligned and floating.

5.
Camera:
Andor Clara: cooled CCD camera. **With higher magnification objectives camera vibration becomes noticeable. To avoid this use the software option that allows turning camera cooling off during acquisition (Auto on/off).** There are two holes at the back of the microscope casing, which contain screws. The one closest to the camera adjusts the camera angle, the other one adjusts the tube length.

How to check the camera angle on assembly:
- Special metal tool inserted into probe slot at 45° angle just like the mirror
- Use transmission light: 760 nm, 150 ms exposure, no filter
- Focus on the right (pointed) edge of the tool using the z control

- Draw an ROI around the edge of the blade or use the cross hairs
- If the blade does not look horizontal on screen loosen screw (1.3 mm) in hole at back of casing closest to the camera, which fixes the camera in place.
- Slowly rotate the camera until the blade in the field of view is perfectly horizontal and fasten screw.
- When you move the blade in +y in 100 μm steps it must not move in x.

The stage movers:
The cables that connect the stage movers (x,y,z) must not hang down and pull. Ensure that the heavy connectors are fixed to the table.

Galvo mirror:
1 screw for alignment, no alignment should be necessary. If there is a problem contact Alexander Atzberger.
(alexander.atzberger@physikalischebiologie.de)

DIA illumination:
Transmission light with power switch set to maximum and therefore fixed intensity, can only be controlled by changing the exposure time and changing the camera bits.

The chamber:
- Finger gloves are available from pharmacies (Latex without powder, size M).
- Coverslips: 300 μm thickness, 18 mm diameter, glued in with nail varnish from the outside
- Grease for the chamber: Glisseal N, Swiss, grease for laboratories suitable for vacuum, without silicone

6.
How to change objectives:
Only change objectives when the microscope is turned off.
- Remove water from chamber, set x stage control to x=0, set z stage control to z=0, set y stage control to y=0.
- Unscrew the chamber.
- Change objectives.
- Check that the finger glove is in the right position and not ruptured. It is better to change the finger glove when changing objectives. You need a special tool to unscrew the rings that hold it in place. Fit a new glove over the inner ring and cut off the finger leaving ca. 1.5 cm. Screw the finger glove in so that the fat end points to the inside of the chamber. Push the cut-off end in as well.
- Screw the chamber back in, fill with water and check for leaks. Check again after 15 min.
- Turn microscope back on and activate Preview in the acquisition software.
- Set the y stage control to y=13,000, set the z stage control to z=-1,500.
- Fit the sample/capillary.
- Set the z stage control to z=0.

7.

How to start the microscope and the acquisition software:

- There is a red start button on the console for the microscope that also controls the realtime computer. The acquisition software computer is separate. **When the microscope crashes press Reset button on the realtime computer (mDSLM-LightMicroscopy Group-EMBL-Heidelberg).**
- Acquisition software computer sign in: your Biochemistry login, password: your Biochemistry password, **(embl, password: mdslm), software: Lsfm Contr 4.9.1**

  Recorded Data
  Set up network path
  Copy only: copies data from computer to network without deleting.
  Save (in folder on computer)
  File name: saves on computer
  File structure:
  Multiplane: 1 Tiff per z-stack and channel and time point
  Hierarchical: 1 folder for timepoint containing 1 folder per specimen
  Flat: 1 folder and every file is in there, 1 file for each plane and each channel
  Format: **Tiff 16 regular**: can contain up to 4 GB
  Big Tiff: can hold bigger files, for sCMOS cameras only
- Scan controller: settings can be saved and loaded, do not touch Gamma and Angle
- **The minimum exposure time is 50 ms** because the scanner moves too slowly for less.
- We will get software upgrades and will be notified by email beforehand.

8.

mDSLM alignment procedure using the mirror:

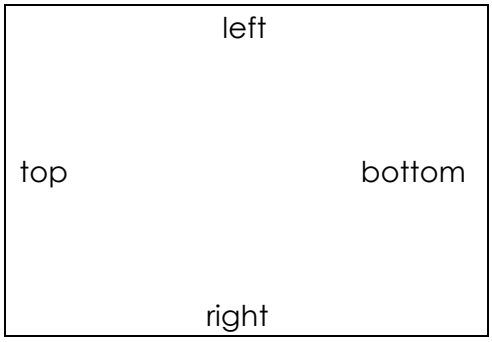
The chamber must be filled with water to above detection lens level.

- Click enter after typing each number to set scan- and Pifoc-parameters to:

  Lightsheet height: 1000
  Illumination Pifoc: 50%
  Detection Pifoc: 50%
  x-centre and y-centre: 0
- Move stage up (using the +y stage control button; x,z and Φ (angle) must be in their default position i.e. at zero) until it couples fully to the specimen holder (at ca. y=10,000 μm) and then lift the specimen holder (by ca. 1,500 μm) again using the +y stage control to a point where it is positioned well above its parking position and where it can now be freely moved in the horizontal (x/z) plane. Now move the specimen holder to the front right end (z=-1,500 μm and x=1,500 μm) of the chamber using the –z and +x stage control. Insert the alignment mirror (using the provided forceps) so that its surface normal (its reflective surface is the one that intersects the centre of the cylindrical rod it is glued to) roughly aligns with the detection axis (mirror surface faces the detection objective). Adjust the insertion height so that the mirror will, when back in the centre position, intersect the illumination beam (centre line of the illumination objective). Move the stage back to z=0 using the +z stage control.

- Turn on the Preview mode with acquisition parameters set to:
  Laser line of your choice
  Power: 0%
  Integration time: 50ms
  No filter
  Now rotate the stage (carrying the specimen holder and the alignment mirror) by 45° using the Φ stage control so that the illumination light is intersected and directed towards the detection objective roughly parallel with the detection axis (if necessary you can also use the x or z stage controls to bring the mirror into that position). If not already visible, increase the power until you see a dim light sheet or light spot on the camera image.
- **10x objective:** Use the x stage controls to bring the light sheet into the centre of the camera image. Set the step-size for the Φ control to 1° and using the +Φ and -Φ stage controls find the angular acceptance limits/range of the detection lens and rotate the mirror to the centre of this range.
- **Higher magnification objectives:** If the above is not possible, i.e. light sheet moves out of the field of view, use detection Pifoc and Φ stage controls to align the rotation stage. When moving the Pifoc the light sheet moves out of focus but also changes position if the mirror angle is not at 45°. Use the Φ stage control to rotate the stage by 1° (either direction is fine) and check by moving the Pifoc over the full range whether the shift has decreased or increased. If it has increased change the direction of the Φ step, otherwise continue in the same direction with another Φ step. Successive steps (minimising the position shift of the light sheet) bring the mirror angle closer and closer to the 45° position.
- The mirror now intersects the illumination and the detection axes by 45±1°.
- Use the detection Pifoc control to bring the mirror surface (not the light sheet) into focus, which, when achieved, is apparent by well resolved (always present) dust particles on the surface of the mirror.
- If this is not possible, i.e. the Pifoc range is not large enough, use the scanner offset control together with the z stage control (use small step sizes, e.g. 10μm) to bring the surface of the mirror within the range of the Pifoc while keeping the light sheet position centred on the camera range.
- Minimize the thickness of the light sheet visible on the mirror, i.e. in the centre of the camera image using the illumination Pifoc control.
- If not already correct, move the light sheet centre along its axis to the centre of the camera image using the scanner offset control (top panel) and adjust the light sheet height to span the whole image or a smaller ROI.
- Remove the alignment mirror using the reverse procedure of fitting it.
- Alignment is finished.
- Specimen can be inserted.

Camera view of the sample



9.

Alignment procedure for several laser lines using the mirror:

(Use the mirror only if you are not successful using beads for alignment. The 488 nm laser line is now too strong to be used with the mirror.) **The mirror is used to correct chromatic aberration between different laser lines. Do the alignment below using the mirror before the bead alignment if you want to image several channels.**

- Click on Preview. Insert the mirror with y=13,000 and z=-1,500, turn to 45° using tweezers, adjust y to right height with tweezers if not inserted into the specimen holder all the way. Then move to z=0.
- Adjust x with the x stage controls until the laser beam is visible (laser line of your choice, integration time: 50 ms, no filter), adjust dynamic range (bits) in between. With the 40x objective only one of the two reflections will be visible. Move the x stage until you see the higher beam and concentrate on that. If the beam is not perfectly horizontal it does not matter and is caused by the mirror not being perfectly vertical. Do not infer that the camera angle is wrong.
- Lower laser intensity to 0.
- Use **y height** to reduce lines to dots (y height changes the height of the light sheet: the higher the light sheet the shorter the time per point, which decreases the laser intensity and increases the length of the lines on screen).
- Use **y center** to change left/right positioning of the dots to the centre of the screen. Y center moves the whole light sheet up or down.
- **Z position** moves the lines up or down. **Do not use!**
- Now use y height to increase the length of the lines on screen so that the ends are outside the field of view and the bright end is not visible anymore.
- Concentrate on the upper line. When moving the **detection Pifoc** a long way the light sheet may move in x. If this happens the mirror is not at precisely 45°. Change Φ in 1° steps and move the detection Pifoc until the light sheet stops moving in x.
- Use the **detection Pifoc** to focus on dirt on the upper light sheet.
- Activate the cross hairs and move the upper light sheet to the centre using the x stage control.

- Use the **illumination Pifoc** to find the position where the light sheet is slimmest.
- Now adjust the second channel by choosing the relevant laser line, no filter and laser power=0.
- The upper light sheet of the first channel has to be in the centre of the cross hairs (see above).
- If the laser power in the second channel is too strong even when using the full dynamic range (14 bit) increase y height temporarily to the highest setting.
- Enable both channels and use **light sheet position offset** to perfectly superimpose both (least amount of wobble, match both intensities as well as possible). It is always best to adjust the outer λ to the position of the 561 nm laser line.
- If you want to adjust three channels set the light sheet position offset of the middle wavelength (561 nm) to 0.
- Move the top line of the light sheet of the 561 nm laser into the centre of the crosshairs using the x stage control.
- Switch channel and move the second laser to the same position using light sheet position offset followed by the third.

10.
mDSLM alignment using beads:
Perform a bead alignment every time you change objectives. If you are not changing objectives perform the alignment once a day. Use **Tetraspec beads in 1% low melting agarose**: 1 μm beads for the 10x and 20x objectives and 200 nm beads for the 40x and 63x objectives.

- The default stage settings before adding the beads are y=0, x=0, z=0, Φ=0.
- Click on preview to activate stage settings and laser. Move the y stage control to y=13,000, then move the z stage control to z=-1,500.
- Add the beads and move the z stage control to z=0.
- Use any laser line, laser power >0, integration time: 50 ms, appropriate emission filter.
- For coarse adjustments go to Settings under Scan Contoller. Use detection Pifoc to get beads in focus and adjust y height as well as y center (can I adjust Y height and center using beads?).
- Use the z stage control to move the light sheet to the edge of the agarose block that is closest to the detection objective.
- Set the correct **z spacing**:
  (Pixel size in x and y x 4)/objective magnification
  (6.45 μm x 4)/10 = 2.58 μm z spacing
  (6.45 μm x 4)/20 = 1.29
  (6.45 μm x 4)/40 = 0.645
  (6.45 μm x 4)/63 = 0.402
- Set Start plane, then set 300 planes by moving the z stage control until you get ca. 300 planes. By clicking on Set End Plane the number of planes is updated.
- Set timelapse: long total duration: 1h, interval: 30 s.
- Untick Save next to Start Acquisition, tick z stack and time lapse and click Start Acquisition
- Use the detection Pifoc to focus beads: strongest signal, no rings.

- Use the illumination Pifoc to move the focussed beads into the middle of the sheet.
- Change the laser line and align again.

11.

How to centre the stage:

This needs to be done when a new chamber is fitted to the microscope. On delivery the stage is centred.

- Remove the chamber and unscrew the basin, put the basin aside
- Unscrew the head of the stage
- Fit the head of the stage to the chamber in the least wobbly position. Make sure that the specimen holder keeps its correct position.
- Put the chamber back in with the head of the stage attached
- The pin attached to the head of the stage will now not be centred to fit the base.
- Using the software move the stage in x and z and later also in y to centre it to the pin.
- Adjust the pin in such a way that it does not touch the sides of the hole.
- The stage connects with the table at y = 10,000. Move to +10,000 and tighten screw.
- To access the microscope controller open http://192.168.1.2. in Internet Explorer
- Remote file browser: button on the left
  Open folder
  Tara Settings
  Stage Settings
- Use Copy and Paste buttons to create a copy of the stage settings
- Double click on the file to save it on the desktop and give it the same name as before
- Open file in Excel if installed
- Home position is the important one:
  old value + movement + new value for the x and y axes
  7800 1100 8900
- Save the file and use download/upload button to move the file to the folder
- Could have used the Edit button for all this instead
- Restart realtime computer by pressing reset button on realtime controller after exiting the acquisition software
- Console button shows what the realtime computer is doing after restarting Internet Explorer
- Restart acquisition software
- Unscrew head of stage again and re-attach it to the table to check if it is centred
- Move the stage up to +10,000 and tighten the screw

12.

Specimen: Multi Stage Position

- Specimen 1
- Define new specimen
  Sync channels: sync basic setup (this will copy stack size and position as well as spacing) or just fill in spacing

- In specimen 1 and then in specimen 2 move to correct position, set start plane set end plane (start at surface and end further into the sample, moving in the other direction is possible).
- Set time lapse (this is valid for both channels).
- Click Save and decide where to save
- Set Position Here: button without function
- Start acquisition
- When finished open file in Explorer
- Open Fiji
- Highlight file in Explorer and drop into Fiji
- Stacks
  Manipulation
  Concatenate
- When you have your concatenated stack:
  Stacks
  Hyperstack
  Stack to Hyperstack
- For z projection over time:
  Image
  Stacks
  z-project

13.
Rotation
There are two tools for rotation compensation: a metal needle for the 10x and 20x objectives and a glass needle for the (20x and) 40x objective. To make the glass needle break a bit off a Femto tip and glue it on to the holder for thin capillaries.
- Fit the needle into the specimen holder (760 nm, 150 ms exposure, filter empty) and centre it using the crosshairs.
- Focus on the needle and then fit an ROI to the tip of the needle.
- Advanced (in top menu)
  Manual Calibration: follow instructions and when finished press Finish
- Remove the needle and insert your sample, focus on it and set up a stack that starts way outside the specimen and ends way outside the specimen.
- Rotation: start angle 0°, end angle 315°, angular step 45°, steps 8 (alternatively: start angle 0°, end angle 330°, angular step 30°)
- Possibly set camera gain
- Tick Z-stack, tick Rotation, tick Time Lapse, tick Save, Start Acquisition

Alternatively: use Next Specimen for all the different angles
Rotation works only if the needle for compensation, the capillary and the agarose are absolutely vertical and if there is no wobble. One should mount samples in micropipettes for the rotation.

14.
Structured Illumination
This generates 3 images per plane, phases are moved by 120°.
- Structured illumination
  Scanning Properties
  Laser Power Modulation

Current SI Frequency: modulates laser on/off, depends on the sample and on how deep you are inside the sample, 30-60 are good numbers

15.
Preparation of Tetraspeck beads for mDSLM

objective lens	final conc. (beads/mL)	bead stock/150 μL	
10x/NA 0.3	3.15E+07	5.19 μL	1 μm beads
20x/NA 0.5	3.45E+08	56.85 μL	1 μm beads
40x/NA 0.75	6.29E+09	40.95 μL	200 nm beads
63x/NA 1.0	2.77E+10	180 μL	200 nm beads

Set the heatblock to 70 °C. Prepare 2% low melting agarose in 2x the buffer you are going to image your samples in. Vortex your bead stock thoroughly and make up your beads in H_2O dest. to a total volume of 75 μL. (For the 63x lens the beads need to be dried down in a speed vac before use.) Warm the diluted beads up in the heat block. Add 75 μL of the low melting agarose and vortex thoroughly. Put back into the heat block and draw the liquid up into the capillary using the Teflon plug. Hold capillary against ice until the agarose is set.

TetraSpeckTM Fluorescent Microsphere Standards
Life Technologies
T7280, 0.2 μm diameter
T7282, 1.0 μm diameter

Samples are mounted in low melting agarose at 0.8 – 1.2%. Cool the agarose down, pipet it into the capillary and add the specimen.
Arabidopsis seedlings:
Grow seedlings vertically on 1% pytagel. Scoop seedling with pytagel into capillary leaving the shoot exposed. Dip bottom end of the capillary in 1% low melting agarose to lift up the root until it is exposed as well. Add carbon pin to support root.

Perfusion system: Ismatec ISM831C
Capillaries: Hilgenberg (ask Alex)